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RESEARCH ARTICLE

Tenderness and physicochemical characteristics of meat treated by recombinant bromelain of MD2 pineapple from a codon-optimized synthetic gene

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ABSTRACT

Bromelain is a complex of cysteine proteases from pineapple (*Ananas comosus*) which was widely used in meat tenderizers. Earlier, using a synthetic optimized gene approach, recombinant bromelain of MD2-pineapple (MD2-MBro) was successfully produced in a fully soluble form. Nevertheless, the use of MD2-MBro to tenderize the meat has never been examined. Indeed, no report on the meat tenderization activity using recombinant bromelain was found. The aim of the current study is to determine the effect of MD2-MBro on meat tenderness and its physicochemical properties. To address this, MD2-MBro was over-expressed in *Escherichia coli* BL21 CodonPlus(DE3), followed by purification using a single step of Ni-NTA affinity chromatography. Fresh lamb shoulder meat from a local market in Kota Kinabalu, Sabah, Malaysia, was then treated with MD2-MBro at the concentration of 0 (B0), 0.01 (B1), 0.05 (B2), and 0.1% (B3). The meat tenderness was measured using Warner-Bratzler shear forces, indicating that the addition of MD2-MBro had significantly ($P < 0.01$) reduced the shear force value from 8.80kg/cm^2 to the range of 6.01 to 6.92 kg/cm^2, which falls under the category of tender. The ability of MD2-MBro to tenderize meat might be related to its ability to degrade myofibril protein, as demonstrated by the formation of a clear zone under an agar plate system and scanning electron microscopy. Besides, the total protein or sarcoplasmic protein solubility was significantly enhanced by the MD2-MBro treatments, along with soluble peptides, free amino acids, collagen content, and collagen solubility, which indicated the improvement in meat protein digestibility. Other physicochemical properties (color, pH, water-holding capacity, and cooking loss) of the meat were affected by MD2-MBro treatments yet remained in the normal range. Altogether, while MD2-MBro consisted of only a single cysteine protease enzyme, this protein can tenderize meat and increase protein digestibility, with acceptable changes in the overall physicochemical properties.

Keywords: Bromelain; Cysteine protease; Meat tenderizer; Recombinant protein; Pineapple

INTRODUCTION

Pineapples (*Ananas comosus*) is known to contain a complex of cysteine proteases which catalyzing the substrates through the catalytic sites of Cys, His and Asn or Glu (Razali et al., 2021; Menard et al., 1991; Otto and Schirmeister, 1997). This enzyme is homologous to papain cysteine protease from papaya (*Carica papaya*) and majorly detected in pineapple's steam and fruit. In addition to being found in the stem and fruit of the pineapple, bromelain has been reported to be present in other parts of pineapple, including crown, peel, leaves, and core (Ketnawa et al., 2012).

The Food and Drug Administration of the United States of America registered bromelain as a safe food supplement and therefore applicable for the food system. A popular application of bromelain in the food system is as a meat tenderizer. The ability of bromelain to tenderize is mainly due to its proteolytic activity in digesting muscle proteins, including myfobril and connective tissue proteins. Practically, meats are seared by thin chunks of pineapple or marinated in blended pineapple before further cooking processes. The use of bromelain as a meat tenderizer is gaining wide interest as tenderness is considered the most important meat quality attribute that affects consumers' perception (Brooks et al., 2000; Morgan et al., 1991; Mennecke et al., 2007). Due to this importance, Bolumar et al. (2013) reported that various studies were conducted to find the best treatments in improving meat tenderness.

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Some studies have shown that fruit or stem bromelain extract can tenderize meat (Ketnawa and Rawdhuen, 2011). The crude bromelain was obtained from pineapple stems or fruits and is further sprinkled on the meat or resuspended for marination purposes. Manohar et al. (2016) showed that the use of pineapple extract containing bromelain was found to increase meat tenderness. A similar report by Singh et al. (2018), and Rani et al. (2022) using the extract from pineapple waste also showed the ability of the extract to improve the meat texture. In general, bromelain and papain were reported to be applicable for different type of meats, including pork, beef, duck, fish and chicken (Chaurasiya et al., 2015; Ionescu et al., 2008; Istrati et al., 2012; Ketnawa et al, 2012; Feng et al., 2017; Buyukyavuz, 2014). Compared to other proteases, especially papain, bromelain is a much more effective meat tenderizer as it doesn't result in a mushy texture or unpleasant off-flavors, which can be observed with the use of papain (Kim and Taub, 1991; Stefanek et al., 2002).

Nevertheless, the challenge of developing this product is consistency in meat tenderness yielded by the bromelain (Bolumar et al., 2013). Noteworthy, as the bromelain works by degrading the muscle proteins, the tenderizing process by bromelain was also reported to affect the other related physicochemical properties of meat, including pH, color, water-holding capacity, cooking loss, and protein quality. This implies that the quality of bromelain for tenderization should then affect the major parameters of the meat quality. To note, previous research on the use of bromelain for meat tenderization mostly used pineapple extract, instead of pure bromelain. The use of pineapple extract has limitation on its consistency as different extract might have different quality and quantity of bromelain (or cysteine proteases). The use of bromelain purified from pineapple waste in meat tenderization was reported by Woinue et al. (2021) which showed its effectiveness in tenderness improvement. The use of pure native bromelain, nevertheless, has some limitations, including the length process of the purification and the consistency of the product quality (Razali et al., 2021). In addition, the scarcity of raw materials for extraction might also be an issue someday due to the trend of agricultural land decreases.

Accordingly, recombinant bromelain is an interesting avenue to explore. Recombinant bromelain refers to a purified form of a single cysteine protease, obtained through overexpression of bromelain gene using bacterial host cells. This contrasts with crude bromelain, which is a mixture containing various proteases. While studies on the incorporation of bromelain extract or pure native bromelain into the physicochemical properties of meats are widely available, to our knowledge, there is no study on the use of recombinant bromelain for this purpose. Furthermore, recombinant proteins often behave differently from their non-recombinant (native) forms (Orlova et al., 2003). This leads to the assumption that the effect of native and recombinant bromelain on the tenderness and physicochemical properties of the meat might be different.

Earlier, we have recently developed a production system that enables us to produce recombinant bromelain from MD2-pineapple (MD2-MBro), which is fully soluble and active forms using *Escherichia coli* host cells (Razali et al., 2021; Razali et al., 2020). The current study is aimed to investigate the effect of MD2-MBro on meat tenderness and other physicochemical properties. In this study, it was demonstrated that, intriguingly, even though MD2-MBro is only a single cysteine protease, this protein could tenderize the meat with acceptable changes in other physicochemical properties. In addition, the protein quality of the meat treated by MD2-MBro was increased. This paves the way for further development of recombinant bromelain as a meat tenderizer.

MATERIALS AND METHODS

Expression and purification of MD2-MBro

The heterologous expression of MD2-MBro was performed according to Razali et al. (2021), where pET-32b(+) containing the codon-optimized synthetic gene of MD2-MBro was transformed into *E. coli* BL21-CodonPlus (DE3) and grown in Luria–Bertani (LB) broth medium at 37 □ in the presence of 25 μg/ml of chloramphenicol and 100 μg/ml of ampicilin. The expression of protein was induced at OD_{600} of 0.6 - 0.7 by adding 1 mM of isopropyl β-D-1-thiogalactosidase (IPTG), followed by further incubation for 3 h at the same temperature, and harvested by a centrifugation at 10,000 g. The harvested cells were then lysed by sonication on ice followed by centrifugation at 30,000 g for 30 min at 4 □ to obtain the soluble fraction, which was then loaded onto a HisTrap FF column for purification, which was pre-equilibrated with 20 mM Tris–HCl pH 8.0 containing 100 mM of NaCl. Elution of the protein was done by imidazole through a linier gradient concentration up to 500 mM. To confirm the purity of MD2-MBro, 15% SDS-PAGE with Coomassie Brilliant Blue R250 stain (Laemmli, 1970) was used to visualize the protein. Meanwhile, concentration of MD2-MBro was determined by using a UV-Vis spectrophotometer at 280 nm based on Goodwin and Morton (1946).

Samples preparation and treatment

The fresh lamb shoulder meat, which was obtained from a local market in Kota Kinabalu, Sabah, Malaysia, was cut and sliced into several pieces of approximately similar sizes

(4.5 cm^3) with the weight of about 3.5 g each (Fig. 1). The meat was divided into four groups: B0 (sample without bromelain), B1 (sample treated with 0.01% of bromelain), B2 (sample treated with 0.05% of bromelain), and B3 (sample treated with 0.10% of bromelain); and marinated for 1 h at room temperature (25 ± 3 □) (Fig. 2). The meat samples were then used for further analysis.

Tenderness

The tenderness level of the sample was determined using Warner-Bratzler shear force based on Warner et al. (2021). The meat samples were first boiled until their internal temperature reached around 80 – 82 □. The meats were then cooled down at room temperature until they reached a constant weight. The meats were then shaped using a 1.27 cm diameter-corer casing to produce unidirectional fiber of the meat. The fiber was then cut by a Warner-Bratzler blade, and the force needed to break down the fiber (kg/cm^2) was recorded. The meats were then categorized based on the results of measurements into very soft (1 – 2 kg/cm^2), soft (3 – 5 kg/cm^2), hard (5 – 9 kg/cm^2), and very hard (more than 9 kg/cm^2) (Nuraini et al., 2013).

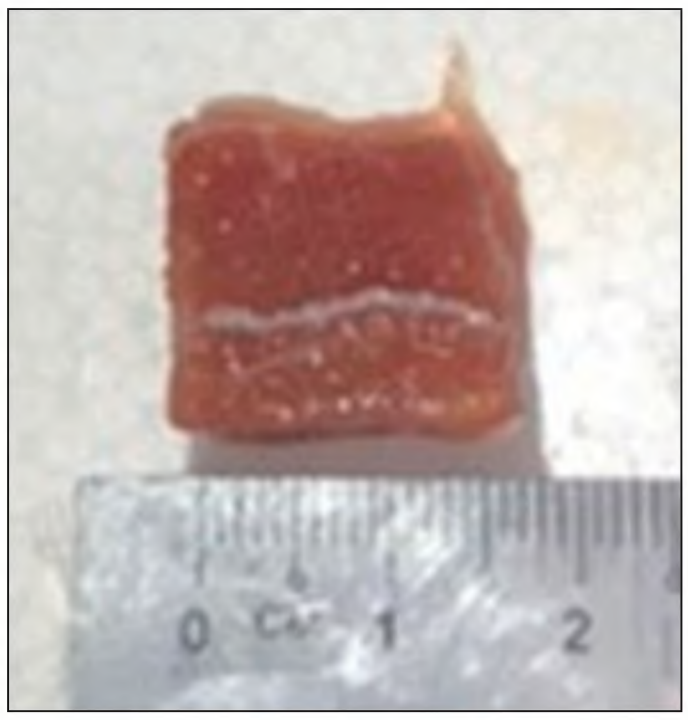

Fig 1. Fresh lamb shoulder meat size for the treatment. The ruler is in cm unit to scale the size of sample.

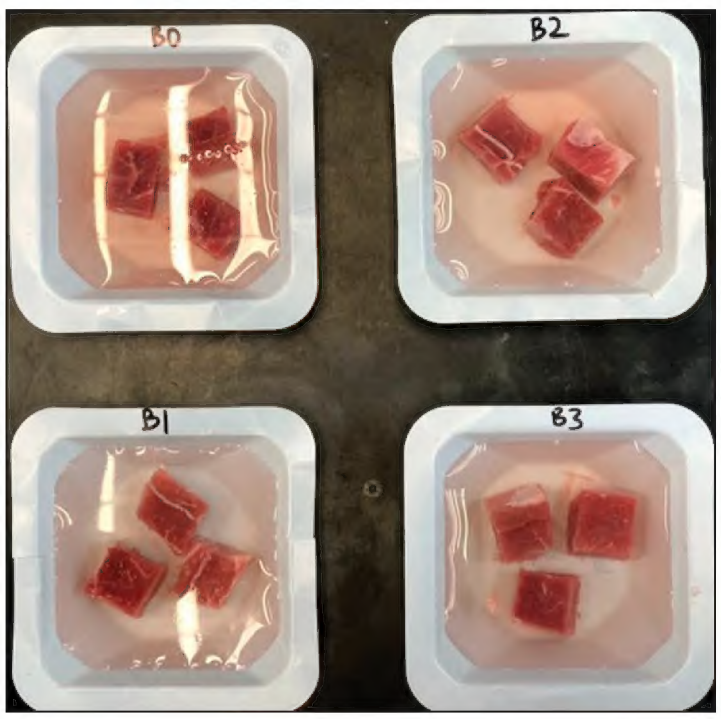

Fig 2. A representative of meat during the treatment. B0 (sample without bromelain), B1 (sample treated with 0.01% of bromelain), B2 (sample treated with 0.05% of bromelain), and B3 (sample treated with 0.10% of bromelain).

Myofibril proteins extraction

The extraction was performed based on the method of Molina and Toldrá (1992) with some modifications. For this purpose, 1 g of sample was used with 0.03 M phosphate buffer (pH 6.5) used to resuspend the buffer. The suspension was homogenized for 4 min using a blender and centrifuged at 10,000 rpm for 20 min at 4 □. The collected pellet was then washed in the same phosphate buffer to remove muscle proteases. Next, the pellet was weighed and resuspended in 0.1 M phosphate buffer, pH 6.5 containing 0.7 M potassium iodide (KI) and 0.02% sodium azide. Once homogenized for 8 min, the mixture was centrifuged again at 10,000 rpm for 20 min at 4 □, and the supernatant was collected.

Scanning electron microscopy (SEM)

The microstructures of the samples were analyzed using a scanning electron microscope (SEM). The muscle specimen preparation was done based on Ketnawa and Rawdkuen's (2011) method with some modifications. The uniform-sized 1 cm^3 of meat shoulder was weighed and marinated in 20 mM phosphate buffer (pH 8.0) as a control and in MD2-MBro protein for 1 h at room temperature. Next, it was rinsed with distilled water and dried on a paper towel. Each sample was then cut perpendicular to the longitudinal orientation of the muscle fibers using a sharp blade. The specimens were observed in a Hitachi S-3400N SEM system (Tokyo, Japan) at an acceleration voltage of 10 kV.

Proteolytic activity of myofibril proteins

The ability of MD2-MBro to degrade myofibril protein was determined using an agar plate system, according to Mauriello et al. (2002). Briefly, 2.5% of Bacto agar with 10% of myofibril protein was autoclaved. The autoclaved agar media were then poured per plate and allowed to harden. Next, wells were made on the plate, and different concentrations of MD2-MBro protein (0, 25, 50, and 100 μg/ml) were filled in the wells. After the placement of each well, the plates were incubated at 37 □ and observed for two days for the halo zone formation.

Total protein and sarcoplasmic protein solubility

Two protein factions (sarcoplasmic and myofibril) were first separated from the sample using the method of Joo et al. (1999). The Lowry method was used to quantify protein concentration. Protein solubility was determined by extracting the sample in pre-cold buffer of 0.1 phosphate buffer (pH 7.2) containing 1.1 M potassium iodide. The steps homogenization, shaking, centrifugation, filtration,

and protein determination procedures as previously described were employed. The solubility of both total and sarcoplasmic protein was reported as mg of protein/g of sample.

TCA soluble peptides and free amino acid contents

TCA-soluble peptides (2015) was determined according to Maqsood et al. (2015). Meat samples (3 g) were homogenized at 19,000 rpm with 27 ml of 5% trichloroacetic acid (TCA) (w/v). The homogenate was stored in ice for 1 h and centrifuged for 5,000 g for 5 min. The Lowry method was used to determine the number of soluble peptides in the supernatant, which was expressed as mg of protein/g sample. The concentration of free amino acids in the dissolved fractions after enzyme hydrolysis is assessed by a ninhydrin test (Murariu et al., 2003).

Total collagen and hydroxyproline content

The hydroxyproline (HP) content in the meat samples was determined using the method suggested by Naveena and Mendiratta (2001). A standard hydroxyproline solution was also included, with concentrations ranging from 10 to 60 ppm. Equation 1 was used to compute total collagen (mg/g sample):

$$Total\ collagen = Hydroxyproline\ content \times 7.14 \quad (1)$$

Collagen solubility

Collagen solubility was determined using the method of Naveena and Mendiratta (2001), which was calculated according to Equation 2 (Williams and Harrison, 1978).

$$Soluble\ collagen\ content = 7.14 \times \%\ HP\ solubilised \quad (2)$$

Meat color

Measurement of meat color was done according to Ergezer and Gokce (2011) using a portable chromameter (CR–400 Minolta, Osaka, Japan) with D65 illuminant and an 8 mm aperture size. The L*, a*, and b* values correspond to lightness, redness/greenness, and yellowness/blueness, respectively, and were measured and used to describe color expression (chromaticity coordinates). The chromaticity coordinates are as follows: ± a* (red direction), –a* (green direction), ± b* (yellow direction), –b* (blue direction) (blue direction). For the measurement, a white reference tile was used for calibration.

pH

The measurement was done according to Manohar et al. (2016) using the CyberScan pH6000 pH-meter equipped with a glass electrode was calibrated using a standard sodium acetate buffer of pH 4.0 and pH 7.0. The mean value of triplicate samples measurements was calculated as the assay results, and pH values were measured for all the samples at room temperature (25 ± 3 □) (Wyrwisz et al., 2012).

Water holding capacity (WHC)

The water holding capacity (WHC) of the lamb shoulder meat was determined according to Tamzil's (2017) procedure. The level of WHC was calculated with Equations 3 and 4:

$$mg\ HO = \left(\frac{Width\ of\ the\ wet\ area\left(cm^2\right)}{0.0948} \right) - 8.0 \quad (3)$$

$$\%\ of\ free\ water = \left(\frac{mg\ HO}{300} \right) \times 100\% \quad (4)$$

Whereby "0.0948 mg H□O" is the constant formula and "300" is the conversion yield of 0.3 g to mg.

Cooking loss

Cooking loss was determined according to the procedure described by Watanabe et al. (2018) with slight modifications. Cooking loss was measured by the weight difference before and after cooking the meat. First, 5 g of meat sample was weighed in a plastic bag and subjected to boiling at 80□ for 1 h. After that, the cooked meat sample was cooled down in a cold room (4 □) overnight (16 h). Then, the sample was weighed again after cooling down. Each measurement was performed in three replications, taking the mean value as the assay result. The percentage of cooking loss was calculated using Equation 5.

$$Cooking\ loss = \left(\frac{Weight\ before\ cooking - Weight\ after\ cooking}{Weight\ before\ cooking} \right) \times 100\% \quad (5)$$

Statistical analysis

The data were expressed as mean ± standard of deviation of three independent replications. The experiment was performed under a Completely Randomized Design (CRD) (Sekhar et al., 2019), with a single factor (MD2-MBro) with four concentration levels (0, 0.01, 0.05, and 0.1%). The differences among the means were statistically analyzed through one-way ANOVA (Analysis of Variance) with Tukey's post hoc test (Midway et al., 2020). The statistical analysis was performed using Minitab Ver. 19.1 (Minitab, L.L.C, USA).

RESULTS AND DISCUSSIONS

Recombinant protein

Fig. 3 showed that MD2-MBro obtained from the current production using *E. coli* cells was in high purity after Ni-NTA affinity chromatography, as demonstrated by the absence of visible contaminant protein bands. The

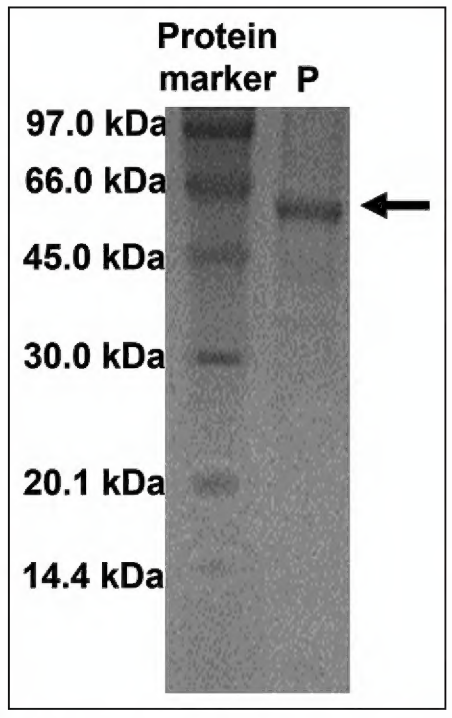

Fig 3. Purified MD2-MBro after Ni-NTA chromatography. The band corresponding to MD2-MBro is indicated by the arrow.

apparent size of MD2-MBro is 56 kDa, which is higher than the calculated size from its amino acid sequence (39 kDa). This is due to the presence of the thioredoxin (Trx) tag at the N-terminal of MD2-MBro, which added about 12 kDa. The tag was earlier reported to assist the solubility of MD2-MBro upon the expression (Razali et al., 2021). The tag was not removed in the current production as we had confirmed that the tag does not affect the proteolytic activity of MD2-MBro (Razali et al., 2020; Razali et al., 2021). As the tenderization process is assumed to be highly associated with proteolytic activity, the presence of the Trx-tag should not lead to the bias issue due to the unspecific effect of the tag. Besides, the removal of the tag should require more steps in purification, which is considered unfavorable, timely, and costly. Earlier, we also found that the tag could slightly stabilize MD2-MBro (Razali et al., 2021). Altogether, keeping the tag in MD2-MBro is considerably worthwhile for a more efficient production process and stability. The current production yielded about 18 mg from 1 L culture, which is comparable to the previous report (Razali et al., 2021).

Effect of MD2-MBro on tenderness

The use of lamb in this study is due to the report that goat/sheep meat is allegedly less tender than beef or chicken meat (Komariah et al., 2009). Table 1 showed the shear force values of lamb with or without recombinant MD2-MBro treatment. ANOVA result shows that the treatments have a significant effect on the shear force value of the meat ($P < 0.01$). The shear force value of the untreated meat (B0) was 9.80 kg/cm^2, which is quite tough. The post hoc test further revealed that the treatment of recombinant MD2-MBro significantly yielded lower shear force values than the control. The shear force values of the treated meats ranged from 6.01 to 6.92 kg/cm^2, which fall under the category of tender, according to Aberle et al. (2001).

Table 1: Shear force values of the meat samples

Meat samples	Tenderness
B0	8.80 ± 0.71^{A}
B1	6.92 ± 1.18^{B}
B2	6.23 ± 0.96^{B}
B3	6.01 ± 0.35^{C}

Different letters following the means represent significant differences at $P < 0.01$

This suggested that recombinant MD2-MBro was able to tenderize the meat, as shown by the lower shear force value. Interestingly, the shear force values among the treatments (B1, B2, and B3) were found to be statistically comparable ($P > 0.05$). This suggested that 0.01% of bromelain (B1) is sufficient to tenderize the meat sample in this study. Nevertheless, descriptively, there was a tendency that the shear values slightly declined along with the increase of MD2-MBro concentration.

It is noteworthy to mention that this study is the first to report on the effect of recombinant bromelain on meat tenderness. Similar results have also been reported earlier whereby non-recombinant bromelains could tenderize meat (Singh et al., 2018; Manohar et al., 2016; Ketnawa et al., 2011; Nadzirah et al., 2016). However, this implies that the current study provides the first evidence of a single cysteine protease (bromelain) member from pineapple exhibiting the ability to tenderize meat. Pineapple extract (non-recombinant bromelain) does not represent a single cysteine protease member as it may contain many types of cysteine protease (bromelain) as reported in MD2-pineapple (Redwan et al., 2016). To note, the concentration of MD2-MBro used in this study was also considerably much lower than the studies on pineapple extract, yet sufficient to tenderize the meat. This might be due to the differences in the specific activity between purified and unpurified bromelain. Srinivasan (1998) proposed that the high purity of protein or enzyme has positively correlated to the specific activity. Thus, it implied that the level of high purity in the enzyme needed to catalyze a reaction should be lower than that of less purity in the enzyme.

Effect of MD2-MBro on meat structure and protein

Noteworthy, the tenderness of the meat is often correlated to muscle structure disruption. Lawrie and Ledward (2006) proposed that myofibril protein degradation should further destabilize protein structure. Therefore, it is important to investigate if the ability of MD2-MBro to degrade myofibril proteins is accompanied by changes in meat structure. Accordingly, analysis of structural changes under SEM and protein contents and solubility were performed.

Scanning electron microscopy (SEM)

Microscopic analysis was conducted to monitor the changes in meat structure in the presence of MD2-MBro.

Fig. 4 and Fig. 5 showed SEM images of the meat without or with MD2-MBro treatment with more than 10 occurrences during the observation from different spots of the specimens. The SEM images showed in Fig. 4, depicting the control samples, demonstrate that most muscle fibers maintained a well-organized structure and closely bound to each other. Meanwhile, the meat treated with MD2-MBro (Fig. 5) exhibited signs of damaged muscle fibers within various bundles, decreased inter-fiber attachment, and disrupted fiber interactions. Additionally, there was fiber disintegration accompanied by a substantial increase in exudates, which resulted in the expansion of the interfibrillar space, or gaps between muscle fibers. These gaps became more apparent as they grew, revealing significant separations between fibers. To note, in this experiment only one concentration of MD2-MBro was used as the purpose was only to confirm the structural changes of the meat upon the bromelain treatment. Ketnawa and Rawdkuen (2011) proposed that these gaps might be due to the degradation of the sarcolemma and endomysial collagen surrounding the muscle fibers. The results also showed that the strong muscle fibers were broken and severely degraded. Muscle fibers are composed of myofibril proteins organized either as thick or thin filaments (Lawrie and Edward, 2006). Accordingly, this suggested that MD2-MBro tenderizes the meat by degrading myofibril meat proteins and muscle fibers. The microstructure level study using SEM by Naveena and Mendiratta (2001) also found broken muscle fibers in different bundles and increased space between the bundles of buffalo muscle when treated with ginger extract. Furthermore, Chen et al. (2006) reported that the protease effect during meat tenderization was not only due to myofibril protein degradation, but also connective tissue degradation.

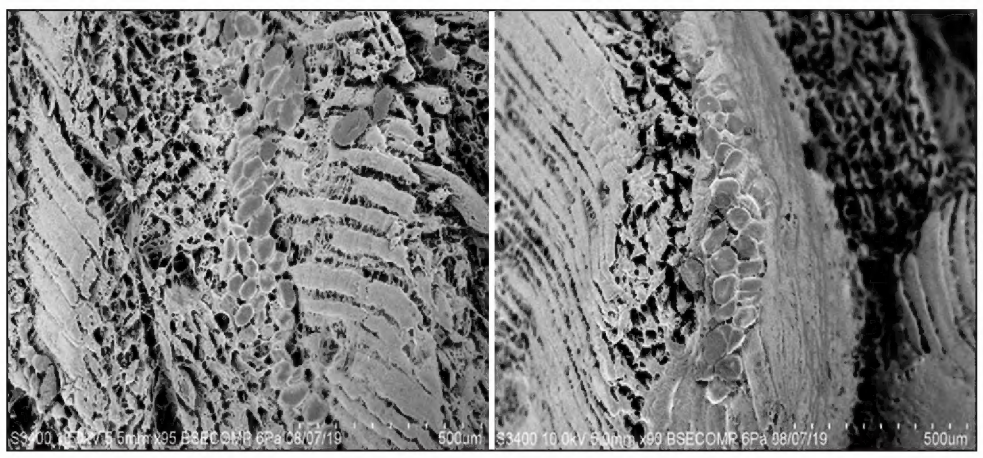

Fig 4. Microstructure of the samples treated without bromelain (control). Magnification of 250x at an acceleration voltage of 10 kV.

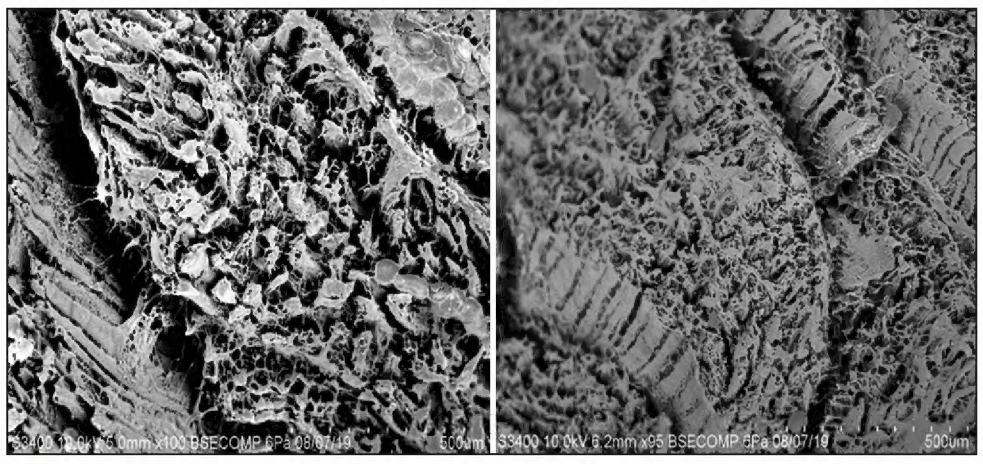

Fig 5. Microstructure of the samples treated with bromelain (MD2-MBro). Magnification of 250x at an acceleration voltage of 10 kV.

Proteolytic activity of myofibril proteins

The effect of MD2-MBro in tenderizing meat is believed to be associated with the ability of this enzyme to degrade muscle proteins, particularly myofibril proteins and connective tissue (Hopkins and Thompson, 2007). Some reports had demonstrated that non-recombinant bromelain exhibited the ability to degrade myofibril proteins (Kim and Taub, 1991; Fang et al., 2017; Ionescu et al., 2008; Maqsood e al., 2018). It is, therefore, interesting to confirm if recombinant bromelain also signifies the ability to degrade myofibril proteins. Fig. 6 showed that MD2-MBro was able to degrade myofibril protein as indicated by the formation of the halo zone. The diameter of the halo zone was increased as the concentration of MD2-MBro increased along the incubation period. Duggleby (2001) reported that time course indeed affected the reaction catalyzed by the enzyme as the longer time course allows the enzyme to interact with the substrate for the catalysis.

Total protein and sarcoplasmic protein solubility

The total protein solubility (TPS) of meat with or without MD2-MBro is shown in Table 2. The TPS values of the treated meat range from 12.01 – 17.31 mg/g of sample,

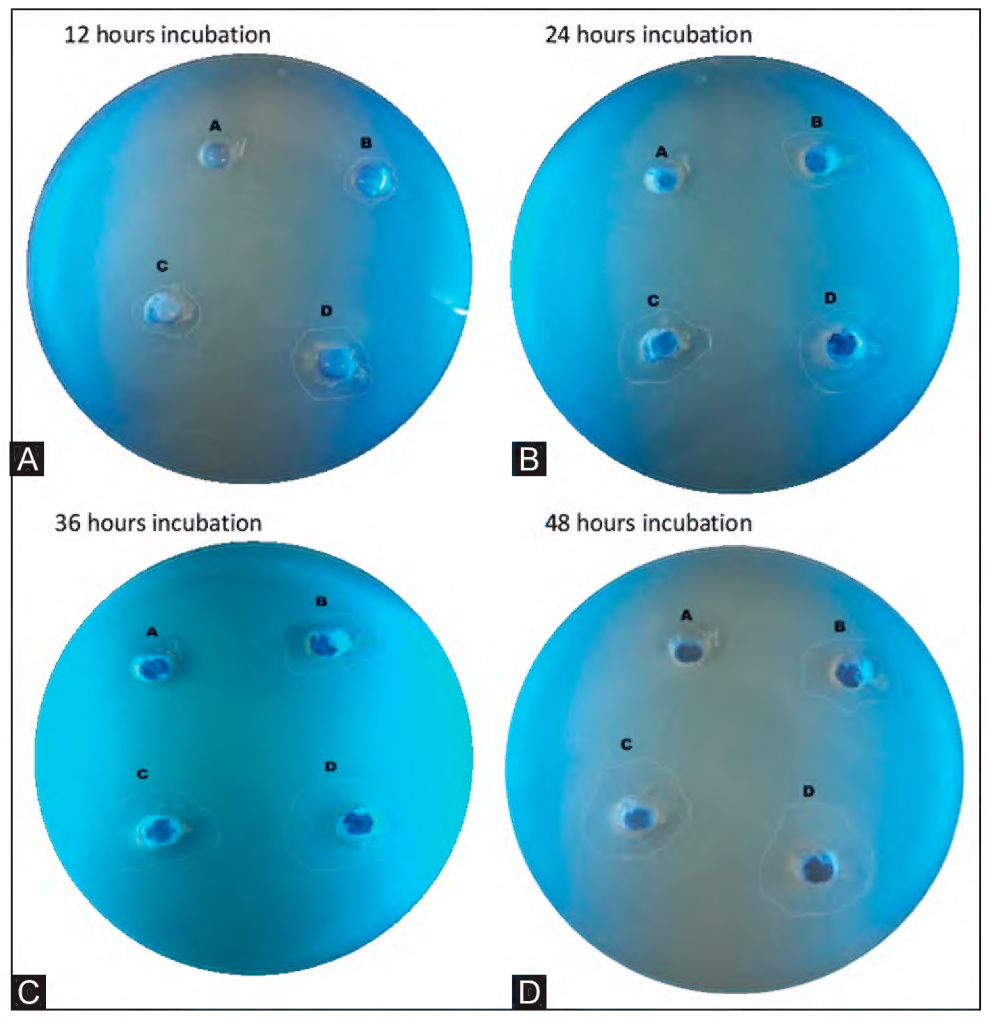

Fig 6. Proteolytic activity of different concentrations of MD2-MBro on 10% myofibril protein plate. A is 20 mM phosphate buffer pH 8.0 (as negative control), B is 25 µg/ml of MD2-MBro, C is 50 µg/ml of MD2-MBro, and D is 100 µg/ml of MD2-MBro.

which is significantly higher than that of untreated meat ($P < 0.05$). The increase in TPS is associated with the ability of recombinant MD2-MBro to degrade myofibril proteins and further increase myofibril permeability. This assumption is in good agreement with a previous report by Rawdkuen et al. (2013). Chen et al. (2017) reported that myofibrillar proteins, which make up 50% of total muscle proteins, are generally considered insoluble in water and require high salt concentration to be solubilized. However, the degradation of these proteins by protease, including bromelain, produced fragments of myofibril that are soluble in water. To note, among several plant proteases commonly used to tenderize meat (ficin and papain), bromelain exhibited the highest degree of myofibril degradation activity (Maqsood et al., 2018). Earlier, Maqsood et al. (2018) also reported an increase in TPS in the meat treated with non-recombinant bromelain. The increase of TPS is a common phenomenon for meat treated by protease, as reported by Naveena et al. (2004) and Naveena and Mendiratta (2001) for hen meat and buffalo meat, respectively, treated by papain.

Similarly, Table 2 also showed that the SPS values of the meat treated by MD2-MBro (ranging from 6.08 to 8.31 mg/g of sample) were significantly higher than that of untreated meat ($P < 0.05$). Sarcoplasmic proteins, mostly glycolytic enzymes, and myoglobin are known to be water-soluble (Malva et al., 2018). Therefore, the presence of protease, including bromelain, would break down the peptide bond of the sarcoplasmic proteins, which are furthermore prone to be reactive to water molecules. Hence, the solubility is increased. Wolfenden (1978) indicated the interaction between peptides and water molecules is mainly facilitated by the free carboxyl group. Notably, Feng et al. (2020) proposed that precipitated or denatured sarcoplasmic proteins might adhere to myofibrils, decreasing the water-holding capacity. Therefore, the degradation of sarcoplasmic proteins by proteases is expected to avoid binding to myofibrils, as the degradation products tend to be soluble in water.

TCA soluble peptides and free amino acid contents

TCA-soluble peptides content of 0.15 mg/ml was found in the untreated sample. The peptides content was significantly increased in the meats treated with MD2-MBro ($P < 0.01$). Similarly, Maqsood et al. (2018), Ketnawa and Rawdkuen (2011) and Singh et al. (2018) reported an increase in peptide contents of the meat treated with non-recombinant bromelain. This result is acceptable as the proteolytic activity of bromelain leads to the degradation of meat proteins, disintegrating and then releasing peptides (Rawdkuen and Benjakul 2012). Ketnawa and Rawdkuen (2011) and Ye et al. (2021) indicated that bromelain's degradation of muscle proteins is mainly observed in myofibril proteins, particularly myosin heavy chains actin and paramyosin. The ability of bromelain to degrade the myofibril protein is not specific for beef only but also for other meats, including duck (Ye et al., 2021), turkey (Doneva et al., 2015), and squid (Xu et al., 2020).

The ability of MD2-MBro to produce more peptides through muscle protein degradation is also accompanied by more production of free amino acids. This argument is in good agreement with the finding of Ketnawa and Rawdkuen (2011). Table 3 indicated that the free amino acid content significantly increased ($P < 0.05$) in the treated meat. A similar finding was also reported by Doneva et al. (2015), Kuzelov et al. (2010), and Nadzirah et al. (2006), who demonstrated the increase of free amino acid content in meats upon the treatment of non-recombinant bromelain. The increase of free amino acids in bromelain-treated meats is advantageous, as it should increase the digestibility of the meat proteins (Kuzelov et al., 2010). Indeed, Nadzirah et al. (2016) highlighted that the total essential amino acid content of bromelain–treated beef was higher than untreated beef.

Total collagen and hydroxyproline content

The total collagen of the meat with and without treatment of MD2-MBro is shown in Table 4. The total collagen of meat treated by recombinant MD2-MBro ranged from 0.37 to 0.81 mg hydroxyproline/g of sample, which is significantly higher ($P < 0.05$) than the untreated meat (0.18). Earlier, Maqsood et al. (2018) also reported a similar result, whereby the total collagen content of the meat increased upon the non-recombinant bromelain treatment. The increase in total collagen content could potentially be attributed to the

Table 2: Total protein solubility (TPS) and sarcoplasmic protein solubility (SPS) of the meat samples

Meat samples	TPS (mg protein/g sample)	SPS (mg protein/g sample)
B0	11.68 ± 0.15^{a}	4.21 ± 0.08^{a}
B1	12.01 ± 0.07^{b}	6.08 ± 0.59^{b}
B2	14.89 ± 1.52^{c}	8.91 ± 0.10^{c}
B3	17.31 ± 0.93^{d}	8.31 ± 0.21^{d}

Different letters following the means in the same column represent significant differences at $P < 0.05$

Table 3: TCA soluble peptides and free amino acid contents of the meat samples

Meat samples	TCA soluble peptides (mg/ml)	Free amino acids (mg/ml)
B0	0.15 ± 0.04^{A}	1.23 ± 0.09^{a}
B1	0.47 ± 0.01^{B}	1.42 ± 0.06^{a}
B2	0.93 ± 0.08^{C}	2.09 ± 0.02^{b}
B3	1.42 ± 0.01^{D}	2.86 ± 0.05^{c}

Different letters following the means in the same column represent significant differences at $P < 0.05$ (small letter) or $P < 0.01$ (capital letter)

degradation of intermolecular cross-linking bonds within collagen fibrils catalyzed by bromelain, thus extracting more collagen during the extraction process. The ability of bromelain to degrade collagen was previously reported by Ha et al. (2012), which demonstrated to have better degradation activity than papain protease. Similarly, Maqsood et al. (2018) reported that the total collagen of the meat treated with non-recombinant bromelain was also higher than that of papain or ficin.

Collagen solubility

As in the total collagen content, the soluble collagen contents of the meats treated by MD2-MBro (0.052 – 0.109 mg hydroxyproline/g sample) were significantly higher ($P < 0.05$) than that of untreated meat (0.034) as shown in Table 5. A similar finding was also reported by Maqsood et al. (2018), where meat treated with non-recombinant bromelain contained higher soluble collagen than the untreated one. The increase of soluble collagen content was not only attributed to the bromelain but also to other proteases used in the meat treatment, including bromelain, ficin, subtilis proteases, papain, alkaline elastase, and bacterial aspartic proteinase (Sullivan and Calkins, 2010; Takagi et al., 1992; Ashie et al., 2002). This indicated that the increase of soluble collagen content is a typical phenomenon observed in meat treated by proteases. The increase in soluble collagen is advantageous, as it is more digestible than its native one (Leon-Lopez et al., 2019).

Table 4: Total collagen and hydroxyproline content of the meat samples

Meat samples	Total collagen (mg hydroxyproline/g sample)
B0	0.18 ± 0.09^{a}
B1	0.37 ± 0.02^{b}
B2	0.52 ± 0.02^{c}
B3	0.81 ± 0.09^{d}

Different letters following the means represent significant differences at $P < 0.05$

Table 5: Collagen solubility of the meat samples

Meat samples	Soluble collagen (mg hydroxyproline/g sample)
B0	0.034 ± 0.0021^{a}
B1	0.052 ± 0.0019^{b}
B2	0.081 ± 0.0010^{c}
B3	0.109 ± 0.015^{d}

Different letters following the means represent significant differences at $P < 0.05$

Effect of MD2-MBro on color

Table 6 showed that the meat treatment with MD2-MBro has significantly increased the meat's L* and b* values but decreased the a* value. This result is in good agreement with Nadzirah et al. (2016) for the round beef treated with non-recombinant bromelain powder. As the a* value refers to the redness of the meat, the decrease of a* value indicated the loss of meat pigment, mainly myoglobin, responsible for the redness color formation. Accordingly, it is understandable that MD2-MBro reduced the redness level due to the degradation of myoglobin by this protease. The possibility of myoglobin degradation by bromelain was previously evidenced by Ye et al. (2021) in duck meat. A similar result was reported by Nadzirah et al. (2016) for the reduction of a* value for the round beef treated with non-recombinant bromelain powder. In addition, Santos et al. (2020) also reported that the marination of beef in a non-recombinant bromelain solution had decreased a*.

Accordingly, it is also acceptable that the L* (lightness) of the meat was significantly increased by the addition of bromelain. The degradation of myoglobin might result in the reduction of the absorption of a dark color (red). In addition, Forrest and Brieskey (2006) added that the changes of L* might be correlated to the pH of meat which further affected the light absorption and reflection on the meat surface. Santos et al. (2020) and Nadzirah et al. (2006) similarly reported that the addition of non-recombinant bromelain increased the L* value of beef. The increase in the L* value was also shown in the duck meat treated with bromelain (Ye et al., 2021).

Further, the b* values of the meat were found to be significantly enhanced by the treatment of MD2-MBro (Table 6). This indicated that the treated meats were more yellowish than the untreated meat. Wang et al. (2019) indicated that the b* value was significantly affected by fat content and oxidation rate, as well as carbohydrate polymerization. Borrajo et al. (2020) and Jo et al. (1999) added that the increase in b* value might refer to the increasing oxidation rate of the fat meat. In this study, MD2-MBro was speculated to increase the oxidation rate due to the decreased fixation of oxygen by myoglobin, which is likely degraded by bromelain. Excessive free oxygen (unbound) should further oxidize the fatty acids of the meat

Table 6: Color of the meat samples

Meat color	Meat samples			
	B0	B1	B2	B3
L* (lightness)	42.57 ± 1.57^{a}	43.86 ± 1.09^{a}	46.03 ± 1.28^{b}	45.98 ± 0.84^{b}
a* (redness)	8.03 ± 0.53^{a}	7.84 ± 0.18^{a}	7.01 ± 0.26^{b}	6.88 ± 0.34^{b}
b* (yellowness)	11.47 ± 0.31^{a}	11.07 ± 0.27^{a}	12.91 ± 0.18^{b}	12.82 ± 0.25^{b}

Different letters following the means in the same row represent significant differences at $P < 0.05$

at a higher rate. The increase of the b* value of the meat upon the treatment of non-recombinant bromelain was reported by Nadzirah et al. (2016), while Santos et al. (2020) indicated that bromelain has no significant effect on the b* value of beef. Interestingly, Ye et al. (2021) showed that the b* value of duck meat was decreased upon the treatment with non-recombinant bromelain. This indicated that the changes in the b* value of the meat might vary. This might be due to differences in their lipid content or indigenous antioxidant level, as supported by Utama et al. (2018).

Effect of MD2-MBro on pH

While it is clear that the effect of MD2-MBro on meat tenderization was indispensable to the event of meat protein hydrolysis, Mir-Bel et al. (2012) reported that the degree of meat protein hydrolysis is also associated with other physicochemical properties of the meat. These include the pH value, cooking loss, and water holding capacity. Table 7 shows the pH values of the meats range from 5.39 to 5.66, which are considered in the normal pH range for the meat (5.40 – 7.00) (Lawrie and Ledward, 2006). ANOVA result revealed that pH values of the treated meat (B1, B2, and B3) were significantly lower ($P < 0.01$) than that of untreated meat (B0). This suggested that the hydrolysis of protein by MD2-MBro promoted pH reduction, which is in good agreement with the studies conducted by Singh et al. (2018) and Ketnawa et al. (2011). Berardo et al. (2015) reported that hydrolysis might release basic amino acids from the parent proteins, increasing pH value. This was evidenced by Nadzirah et al. (2016) on the increased pH value of beef cut after being treated with purified bromelain from pineapple crown. Nevertheless, hydrolysis might also produce hydrogen ions and an acid group of amino acids which further lower the pH value. Accordingly, the balance of ion hydrogen, acid, and basic amino acids production upon the meat protein hydrolysis determines the final pH value. As the pH values of treated meats were significantly lower than that of the control, this suggested that the hydrolysis event by MD2-MBro released more hydrogen ions and acidic amino acids than basic amino acids.

Effect of MD2-MBro on water holding capacity (WHC)

Water holding capacity (WHC) values (Table 8) of the meat with and without MD2-MBro range from 37.97 – 43.28% mg·H_2O. Statistical analysis further showed that the treatment has significantly affected the WHC value ($P < 0.05$). Further, the post hoc test showed that the WHC value of the control meat (B0) was remarkably lower compared to the treated meat (B1, B2, and B3). Meanwhile, WHC values of B1, B2, and B3 were found to be statistically comparable ($P > 0.05$). This pattern is similar to the effect of MD2-MBro on shear force value which further suggested that the WHC value of the meat is confirmed to be associated with the degree of protein hydrolysis event that occurred in the meat system. To note, WHC is defined as the ability of meat and meat products to bind water (Pearce et al., 2011). It is considered an important parameter as it determines the visual acceptability of the meat and eating quality juiciness (Warner, 2017). Degradation of protein, therefore, should produce more water molecules as the binding ability is disrupted (low WHC value). Nadzirah et al. (2016) and Ketnawa et al. (2011) also reported that the addition of non-recombinant bromelain extract significantly reduced the WHC of the meat. Besides, the decrease in WHC value might also be associated with the decrease in the pH value of the meat. Table 7 showed that the meats treated by MD2-MBro have a remarkably lower pH than the control. A lower pH value might promote further protein degradation, which releases the water to the free forms (Joo et al., 1999).

Effect of MD2-MBro on cooking loss

One of WHC's phenotypes is cooking loss which is described as the number of water molecules during the cooking process as indicated by the reduction in meat weight (Drummond and Sun, 2005). Table 9 shows the cooking loss of the meat with or without MD2-MBro treatment. Statistical analysis showed that the treatment significantly affected the cooking loss of the meat ($P < 0.05$), whereby the higher concentration of MD2-MBro yielded a higher cooking loss of the meat as indicated by the higher amount (%) of the released water. This might be correlated with the events of the myofibril protein degradation and WHC

Table 7: pH values of the meat samples

Meat samples	pH
B0	5.66 ± 0.09^{a}
B1	5.48 ± 0.10^{b}
B2	5.42 ± 0.12^{b}
B3	5.39 ± 0.17^{b}

Different letters following the means represent significant differences at $P < 0.05$

Table 8: Water holding capacity values of the meat samples

Meat samples	Water holding capacity (% mg·H_2O)
B0	43.28 ± 0.17^{A}
B1	41.19 ± 0.50^{B}
B2	39.27 ± 2.81^{B}
B3	37.97 ± 4.07^{B}

Different letters following the means represent significant differences at $P < 0.01$

Table 9: Cooking loss values of the meat samples

Meat samples	Cooking loss (%)
B0	32.60 ± 4.61^{B}
B1	37.74 ± 1.72^{AB}
B2	39.10 ± 1.09^{A}
B3	41.44 ± 2.64^{A}

Different letters following the means represent significant differences at $P < 0.01$

changes. Nadzirah et al. (2016), Singh et al. (2018), and Ketnawa et al. (2011) also reported a similar relationship whereby the higher concentration of extract bromelain resulted in higher cooking loss. The cooking loss values were ranging from 32.60 – 41.44% which were still in the common range of meat cooking loss as reported by Lawrie and Leward (2006). Indeed, the cooking loss greatly varied depending on the pH, length of the sarcomere, length of the muscle filament, contraction state of the myofibril, sample weight, and surface area of the sample. Interestingly, the post hoc test revealed that the significant change in the cooking loss was observed to have a concentration of 0.01% and above (B2 and B3). Meanwhile, the meat treated with 0.01% MD2-MBro (B1) concentration was statistically comparable to that of the control (B0). This, however, slightly differed compared to the other parameters (shear force, pH, and WHC), whereby all treated meats (B1, B2, and B3) were found to be statistically different as compared to the control (B0).

CONCLUSION

MD2-MBro treatment remarkably increased meat tenderness, which is likely to be associated with the ability of MD2-MBro in degrading the muscle structure, particularly the myofibril proteins. Furthermore, the treatment of meat by MD2-MBro also increased the solubility of proteins and peptide contents, which indicated that the treated meat is considerably better for digestion. Nevertheless, the treatment was also found to significantly affect other physicochemical parameters (pH, color, WHC, and cooking loss) yet remain in the normal range and it is considered acceptable. Accordingly, the addition of MD2-MBro is considerably effective as a meat tenderizer with no undesirable side effects on the physicochemical properties of the meat and possibly improves protein digestibility. Economically, 0.01% of MD2 was the most efficient concentration as it was able to tenderize the meat with better protein digestibility than the untreated meat with minor changes in its physicochemical parameters.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Authors' contributions

Rafida Razali prepared the initial manuscript, performed the experiments, and data analysis. Vijay Kumar was supervising the student and data analysis and curation as well as finalizing the revised article. Cahyo Budiman was the corresponding author, conceived, designed the experiments, data analysis and provided guide in collected data. All authors discussed the result and contributed to the final manuscript.

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